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ORIGINAL ARTICLE

L-arginine ameliorates experimental autoimmune myocarditis by maintaining extracellular matrix and reducing cytotoxic activity of lymphocytes

Taka-aki Okabe, Miki Hattori, Zuyi Yuan and Chiharu Kishimoto
Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan

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Correspondence:
Chiharu Kishimoto MD, PhD
Department of Cardiovascular Medicine
Graduate School of Medicine
Kyoto University
54 Kawaracho
Shogoin, Sakyo-ku
Kyoto 606-8507, Japan
Tel.: +(81) 075 751 3197
Fax: +(81) 075 751 4281
E-mail: kkishi@kuhp.kyoto-u.ac.jp

Summary

It was previously shown that administration of the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) aggravated murine viral myocarditis by increasing myocardial virus titres. Experimental autoimmune myocarditis in mice and rats mimics human fulminant myocarditis. The effects of L-arginine, a precursor of nitric oxide, upon heart failure in experimental autoimmune myocarditis were evaluated. Dietary L-arginine (L-arginine group) and L-arginine plus N^G-nitro-L-arginine methyl ester (L-arginine + L-NAME group) were administered to C57BL/6 mice immunized with porcine cardiac myosin over 3 weeks. An untreated myocarditis group was prepared. Cardiac damage was less in the L-arginine group compared with the other two groups, as was incidence of heart failure. In addition, extracellular matrix change was less prominent in the L-arginine group. Plasma concentrations of nitric oxide were elevated in the L-arginine group. Cytotoxic activities of lymphocytes were lower in L-arginine group than in other two groups. L-arginine treatment may be effective in preventing the development of heart failure in experimental myocarditis by maintaining extracellular matrix and reducing the cytotoxic activity of lymphocytes.

Keywords
autoimmune myocarditis, extracellular matrix, heart failure, L-arginine, nitric oxide

Myocarditis is caused by many agents, such as viruses, bacteria and drugs (Liu & Masun 2001). The disease is characterized by acute and chronic phases. In humans, acute myocarditis is a potentially lethal disease and precedes the development of cardiomyopathy (Kawai 1999; Liu & Masun 2001). Autoimmune giant cell of myocarditis in rats and mice mimics human fulminant myocarditis in the acute phase (Kodama *et al.* 1990).

Nitric oxide (NO) is a free radical molecule that mediates viral pathophysiological functions and non-specific immunity (Moncada 1992; Moncada & Higgis 1993). Many cell types are capable of producing nitric oxide through the enzymatic conversion of L-arginine to L-citrulline by nitric oxide synthase (Moncada & Higgis 1993). We have previously demonstrated that inhibition of nitric oxide synthase (NOS) increased myocardial virus titres with the aggravation of cardiac pathology in the acute stage of viral myocarditis (Hiraoka *et al.* 1996). In the chronic stage, it induced severe cardiomyopathic lesions (Hiraoka *et al.* 1996). Thus, NO plays a decisive role in the pathophysiology of myocarditis.

In the current study, we evaluated the effect of L-arginine, a precursor of NO, upon acute murine myocarditis with an autoimmune aetiology by the analysis of cytotoxic assay of T cells.

Materials and methods

Immunization

Acute experimental autoimmune myocarditis (EAM) was induced in 4-week-old C57BL/6 mice by subcutaneous injection of 0.2 ml portion of cardiac myosin (10 mg/ml, Sigma, Tokyo, Japan), mixed with an equal volume of Freund's complete adjuvant (FCA), supplemented with *Mycobacterium tuberculosis* H37Ra (Difco, Tokyo, Japan), in the foot pads on days 1 and 8, as previously described (Shioji *et al.* 2000, 2001). Control mice were immunized with FCA alone. The animals were observed up to 21 days. The day of injection was designated day 1.

Medical treatment

To analyse the effect of L-arginine in the mice with or without EAM, the animals were divided into three groups and treated with (i) either vehicle (saline, $n = 12$), (ii) L-arginine (2.25%, L-arginine group, $n = 13$; Sigma) and (iii) L-arginine plus N^G-nitro-L-arginine methyl ester (L-NAME, 100 μg/ml) (L-arginine + L-NAME group, $n = 13$) orally via drinking water for 3 weeks. The mice consumed the drinking water, and daily fluid consumption was monitored. This route of administration has already been reported (Hiraoka *et al.* 1996). Five mice in each group on day 10 were sacrificed and the hearts were removed for the pathological study. Mice were observed daily. At the end of the experiment, mice were sacrificed by bleeding from retro-orbital plexus under light anaesthesia. Serum nitric oxide levels were determined. The organs were weighed and the ratio of organ to body weight was calculated. The organs (lungs, liver and heart) were processed for pathological study and the spleens for cytotoxic assay.

In parallel with the immunization protocol, additional control groups of unimmunized mice (untreated, L-arginine treated and L-arginine + L-NAME treated; each $n = 3$) were also studied for 21 days.

We performed animal experiments in accordance with the Declaration of Helsinki, and these were approved by our institutional ethics committee for animal experiments.

Pathological examination

At sacrifice, macroscopic findings of myocarditis were graded on a scale of 0–4, as previously described (Shioji *et al.* 2001; Nimata *et al.* 2005; Yuan *et al.* 2005). Tissues were processed by standard methods, embedded in paraffin, cut into 5 and 12 μm sections and stained with haematoxylin–eosin and silver impregnation. A silver impregnation method was used to demonstrate the abnormalities of the extracellular matrix (Lesile *et al.* 1990; Cohen-Gould *et al.* 1992; Takada *et al.* 1997; Kishimoto *et al.* 1998, 2001), that is, thick sections (12 μm) were oxidized in potassium permanganate, bleached in oxalic acid, and sensitized in 2.5% ferric ammonium surfate before being impregnated with ammonical silver. The sections were then reduced in 10% aqueous formalin, toned in 0.1% gold chloride, and fixed in 5% sodium thiosulfate. This method stains cardiac connective tissue fibers, ranging in diameter from 0.2 to several micrometers. This connective tissue architecture is not visualized by conventional methods. Myocardial lesions (infiltration, necrosis and fibrosis) were microscopically graded on a scale of 1+ (mild) to 4+ (severe), as previously described (Cohen-Gould *et al.* 1992; Takada *et al.* 1997; Kishimoto *et al.* 1998, 2001; Shioji *et al.* 2001; Nimata *et al.* 2005; Yuan *et al.* 2005). The degree of connective tissue abnormalities was determined semi-quantitatively (Cohen-Gould *et al.* 1992; Takada *et al.* 1997; Kishimoto *et al.* 1998). The other organs were evaluated for evidence of any pathological lesions.

Serum nitric oxide assay

Nitric oxide was measured by Griess method (Green *et al.* 1982). In brief, blood was collected in heparinized tubes and centrifuged. The plasma fraction was diluted 1:1 with nitrite-free and nitrate-free distilled water. The diluted sample was passed through a copper-plated cadmium column to reduce nitrate to nitrite and then reacted with Griess reagents. Absorbance at 540 nm was measured and represents the total amount of plasma NO and products (nitrate plus nitrite).

Cytotoxic assay

This protocol was conducted to measure cytotoxic activity of splenic lymphocytes against EL-4 tumour cells by ^{51}Cr-release assay.

EL-4 tumour cells, benzo(a)pyrene-induced lymphoma of C57BL/6 mice, were used for assay of cytotoxic activity. The cells were so-called natural killer cell resistant and maintained in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS).

Spleens were mechanically dissociated and the cell suspension was collected. Erythrocytes were lysed and macrophages were removed. The cell suspensions were carefully overlayered on Ficoll-paque (Pharmacia, Inc., Stockholm, Sweden) and were centrifuged. The cells residing on the interface were collected, washed and resuspended in RPMI-1640 medium with 10% FCS.

Target cells (1×10^6) were labelled with 3.7 MBq ^{51}Cr for 60 min at 37 °C in 5% CO_2. Labelled target cells were washed three times, and 1×10^4 cells/well were added to flat-bottomed 96-well microtitre plates (Corning) with effector cells at effector/target cell (E/T) ratios of 50/1 and 100/1. After 4 h of incubation at 37 °C, the supernatant was collected using the supernatant collecting system (Skatron, Tokyo, Japan) and the amount of ^{51}Cr released into the supernatant fluid was measured. The percentage of cytotoxicity was calculated using the formula:

$$\%\text{Cytotoxicity} = \frac{(E - S)}{(M - S)} \times 100$$

where E is the counts per min (cpm) released in the presence of effector cells, S is the spontaneous cpm released from target cells incubated alone in medium and M is the maximal cpm released from target cells incubated with 2% Triton X-100. All experimental data are the mean of duplicate or triplicate samples.

Statistics

All values were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA), followed by Fishers protected least significant difference test, was performed. A value of $P < 0.05$ was considered statistically significant.

Results

Pathological examination (Figures 1 and 2, Tables 1 and 2)

At sacrifice, pleural effusion was macroscopically less in the L-arginine group compared with the other two groups. There was no significant difference in body weight or in the ratio of lung weight to body weight among the three groups. The ratios of heart weight and liver weight to body weight were significantly less in the L-arginine group than in the other two groups. The results may reflect less severe myocardial damage in the L-arginine-treated animals. Pulmonary and liver congestion was microscopically less severe in the arginine group compared with the other two groups.

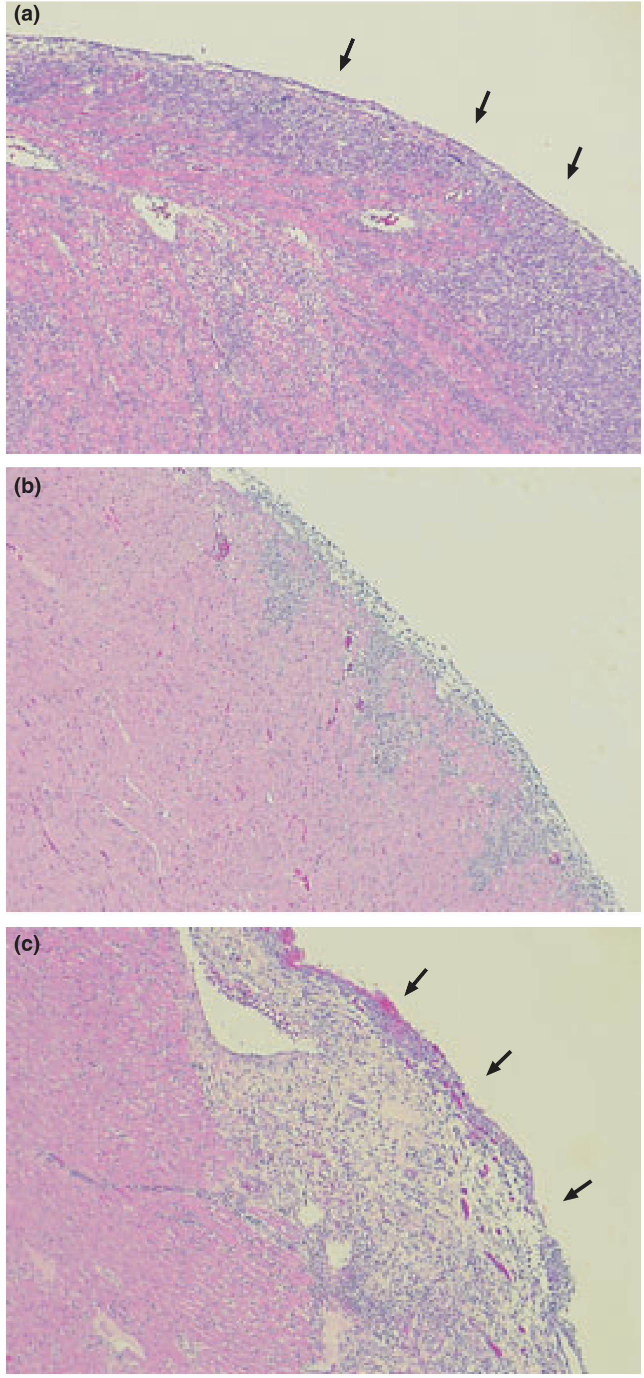

Figure 1 Histological findings in mice with EAM on day 21. Marked diffuse myocyte necrosis and cellular infiltration (arrows) were demonstrated in the untreated mouse (a) and the L-arginine plus L-NAME-treated mouse (c). In the mouse treated with L-arginine (b), myocardial necrosis and cellular infiltration were minimal. Administration of L-arginine (b), but not L-arginine plus L-NAME (c), improved cardiac pathological findings of EAM compared with the untreated control (a). Haematoxylin–eosin stain (×50).

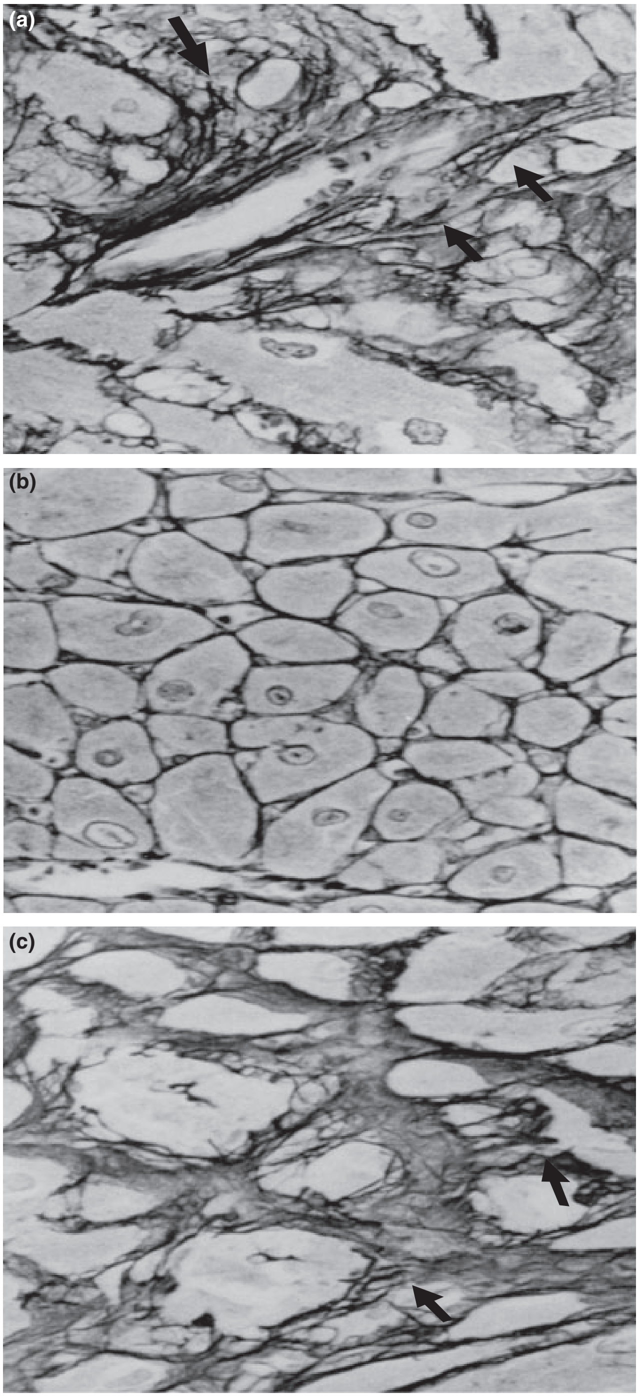

Figure 2 Photomicrographs showing cardiac connective tissue findings. Disorganized, disrupted reticulin fibres nearby necrotic myocytes (arrows) were demonstrated in the untreated mouse (a) and the L-arginine plus L-NAME-treated mouse (c). In the mouse with EAM treated with L-arginine, the architecture of reticulin fibres was less affected (b) than in the untreated mouse (a) and the L-arginine plus L-NAME-treated mouse (c), in which reticulin fibres were abnormally affected. Silver impregnation (a,c × 500; b × 320).

In the untreated and the L-arginine plus L-NAME-treated mice, severe myocyte necrosis with cellular infiltration was noted. However, myocardial lesions (necrosis and fibrosis) were less severe in the L-arginine than in the other two groups. There were no significant changes in the cardiac pathological scores between the L-arginine plus L-NAME group and the control group. Connective tissue abnormalities were less severe in the L-arginine group than in the other two groups. Disorganized, disrupted reticulin fibres nearby necrotic myocytes were marked in the untreated and L-arginine plus L-NAME groups. However, the architecture was less affected in the L-arginine group.

Serum nitric oxide

Nitric oxide was significantly ($P < 0.05$) increased in the L-arginine group (22.7 ± 5.8 μmol/l, $n = 5$) compared with the control and L-arginine + L-NAME groups (control group, 12.2 ± 3.8 μmol/l, $n = 5$; L-arginine + L-NAME group, 17.7 ± 5.6 μmol/l, $n = 5$).

Cytotoxic activities of lymphocytes (Table 2)

Cytotoxic activities of lymphocytes were significantly lower in the L-arginine group than in other two groups.

Unimmunized groups

Pathological examination revealed no abnormalities in the myocardium in either group.

Discussion

In the current study, we demonstrated that oral L-arginine treatment, but not L-arginine plus L-NAME treatment, ameliorated the cardiac pathology of myocarditis in EAM in mice due to maintenance of the extracelluar matrix and reduction in cytotoxic activities of lymphocytes. In addition, long-term oral administration of L-arginine increased serum NO, which suggested that the effects were due to the NO system. Thus, L-arginine may be effective for the prevention against the development of myocarditis with an autoimmune origin.

Nitric oxide appears to play an important role in protecting the myocardium against inflammation (Moncada 1992; Moncada & Higgis 1993). There is evidence that NO can inhibit myocardial remodelling (Khaidar *et al.* 1994). We have previously demonstrated that administration of nitric oxide synthase inhibitor, L-NAME, aggravated coxsackievirus B3 myocarditis in mice (Hiraoka *et al.* 1996) by

Table 1 Organ weights

Group	Treatment	BW (g)	HW/BW ($\times 10^{-3}$)	LuW/BW ($\times 10^{-3}$)	LiW/BW ($\times 10^{-3}$) (mean ± SD)
1, *n* = 12	No treatment	16.8 ± 1.7	10.9 ± 1.5	13.0 ± 2.4	60.5 ± 7.2
2, *n* = 13	L-Arginine	17.3 ± 1.5	8.5 ± 1.4*	11.9 ± 1.8	52.0 ± 5.0*
3, *n* = 13	L-Arginine + L-NAME	17.7 ± 1.4	10.1 ± 1.2	12.4 ± 1,9	57.3 ± 8.7

BW, body weight; HW, heart weight; LuW, lung weight; LiW, liver weight.
At the end of experiment, the ratios of organ weight to body weight were calculated. HW/BW and LiW/BW were less in the L-arginine group compared with the other groups.
*$P < 0.05$ *vs*. control (no treatment).

Table 2 Results of cardiac pathology and cytotoxic activity

		Cardiac pathology (+1 to +4)					Cytotoxic activity (%)	
		Day 10		Day 21			(E/T ratio)	
Group	Treatment	Infiltration	Necrosis	Infiltration	Necrosis	Fibrosis	50:1	100:1
1 (*n* = 12)	No treatment	2.0 ± 1.5 (*n* = 5)	1.6 ± 1.0	2.5 ± 0.6 (*n* = 7)	2.8 ± 0.7	1.9 ± 0.6	18.0 ± 7.32 (*n* = 5)	5.3 ± 9.6
2 (*n* = 13)	L-arginine + L-NAME	1.5 ± 1.2 (*n* = 5)	1.3 ± 0.8	1.6 ± 0.6* (*n* = 8)	1.9 ± 0.5*	0.8 ± 0.7*	8.2 ± 5.7* (*n* = 5)	13.7 ± 7.1*
3 (*n* = 13)	L-arginine	2.0 ± 1.3 (*n* = 5)	1.7 ± 1.1	2.4 ± 0.7 (*n* = 8)	2.5 ± 0.7	2.0 ± 0.8	15.6 ± 9.2 (*n* = 5)	16.2 ± 8.7

Values are given as (mean ± SD). Cytotoxic activities of lymphocytes were significantly lower in the L-arginine group than in other two groups. *$P < 0.05$ *vs*. control (no treatment).

increasing myocardial coxsackievirus B3 titres. Murine EAM is a well-known animal model of myocarditis with an autoimmune aetiology (Kodama *et al.* 1990; Shioji *et al.* 2000, 2001; Nimata *et al.* 2005; Yuan *et al.* 2005). Myocarditis with autoimmune mechanisms might be thought to precede the development of dilated cardiomyopathy (Kawai 1999; Liu & Masun 2001). Most recently, it was demonstrated that L-arginine, the precursor of NO, regulates anti-inflammatory process via regulation of antigen presentation and T-cell system (Rothernberg *et al.* 2006). Accordingly, we examined the effects of dietary L-arginine on the established model of T-cell dependent and autoimmune myocarditis in mice. As a result, treatment with L-arginine, but not with L-arginine plus L-NAME, ameliorated myocarditis associated with reduced cytotoxic activities of lymphocytes, resulting in less left ventricular damage.

In a number of pathological processes which result in ventricular remodelling, inflammatory changes precede the development of connective tissue abnormalities. We used a silver impregnation technique to determine the sequential remodelling of the extracellular matrix that formed in the myocardium in response to active myocyte necrosis (Lesile *et al.* 1990; Cohen-Gould *et al.* 1992; Takada *et al.* 1997; Kishimoto *et al.* 1998, 2001). Interstitial oedema occurs as an early event in inflammatory diseases and is followed by the development of interstitial fibrosis (Cohen-Gould *et al.* 1992; Takada *et al.* 1997; Kishimoto *et al.* 1998). Based on the previous reports (Lesile *et al.* 1990; Cohen-Gould *et al.* 1992; Khaidar *et al.* 1994; Takada *et al.* 1997; Kishimoto *et al.* 1998, 2001), elevated nitric oxide produced from oral L-arginine may suppress the initiation of interstitial oedema, thus preventing the subsequent interstitial fibrosis. The pathological results obtained in the current study may support this scenario, i.e. L-arginine treatment, but not L-arginine plus L-NAME treatment, decreased connective tissue abnormalities and inhibited the ventricular remodelling.

Many mechanisms have been proposed for the protective role of NO in myocardial damage. Nitric oxide inhibits leukocyte and lymphocyte adhesion and chemotaxis, and suppresses the local expression of inflammatory cytokines (Moncada & Higgis 1993). Also, it regulates vasomotor tone which has been reported to be abnormal in heart failure (Moncada & Higgis 1993). A mechanism by which NO may inhibit proliferation of extracellular matrix had also been proposed (Moncada & Higgis 1993).

There are three forms of NOS (Moncada & Higgis 1993). The endothelial (eNOS or NOS1) and neuronal (nNOS or NO3) forms produce the amount of NO required for physiological functions. The cytokine-inducible form (iNOS or NOS 2) is activated by a number of immunological stimuli, such as tumour necrosis α and interferon-γ, and catalyses a high output of NO. It was reported that NO selectively enhances type 1 helper T (Th1) cell differentiation and expansion (Niedbala *et al.* 2007). Th1 cells are key players in the host immune defense against pathogens. A potential candidate for this NO-Th1 self-amplification cycle would be the so-called regulatory T cells ($CD4^+$, $CD25^+$), as mentioned latter (Niedbala *et al.* 2007).

Recently, impaired arginine transport and a relative deficiency of L-arginine in human heart failure were reported (Kaye *et al.* 2000). Most recently, the significant anti-inflammatory role of arginine in inflammatory diseases was also reported, as mentioned before (Rothernberg *et al.* 2006). In addition, it is shown that NO induces the regulatory T cells ($CD4^+$, $CD25^+$), which have the potential to control or regulate the abnormal immune balance in various autoimmune diseases (Sakaguchi 2005; Sakaguchi *et al.* 2006; Niedbala *et al.* 2007; Ono *et al.* 2007). Indeed, in the current study, cytotoxic activities of T cells in the L-arginine-treated group were less associated with higher NO levels compared with the other two groups. Taking altogether with these data, NO itself is beneficial for the prevention against the development of autoimmune-mediated inflammatory myocardial disease via anti-inflammatory, immunomodulating and cardioprotective effects.

In conclusion, oral administration of L-arginine may prevent the myocardial damage in EAM in mice. Exploration of the therapeutic usefulness of oral L-arginine treatment in inflammatory heart diseases appears warranted.

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